Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 1361–1371
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

In vitro effects of mangiferin on superoxide concentrations and expression of the inducible nitric oxide synthase, tumour necrosis factor-α and transforming growth factor-β genes

José Manuel Leiro[a,*], Ezequiel Álvarez[b], Juan Alberto Arranz[a], Isabel González Siso[c], Francisco Orallo[b]

[a]*Departamento de Microbiología y Parasitología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[b]*Departamento de Farmacología, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[c]*Laboratorio de Bioquímica, Departamento de Biología Celular y Molecular, Facultad de Ciencias, Universidad de La Coruña, Coruña, Spain*

Received 23 September 2002; accepted 17 December 2002

Abstract

This study investigated the effects of the natural polyphenol mangiferin (MA) on superoxide anion (O_2^-) production, xanthine oxidase (XO) activity, vascular contractility, inducible nitric oxide synthase (iNOS) mRNA levels, tumour necrosis factor-alpha (TNF-α) mRNA levels, and tumour growth factor-beta (TGF-β) mRNA levels. O_2^- was generated by the hypoxanthine–xanthine oxidase (HX–XO) and phenazine methosulphate (PMS)–NADH systems. XO activity was determined by measurement of uric acid production with xanthine as substrate. Vascular contraction experiments were performed with intact rat aortic rings. iNOS, TNF-α and TGF-β gene expression in rat macrophages stimulated *in vivo* with 3% thioglycollate and *in vitro* with 100 ng/mL lipopolysaccharide and 10 U/mL of interferon-gamma were evaluated semiquantitatively by the retrotranscriptase–polymerase chain reaction. MA at 10–100 μM, like the known O_2^- scavenger superoxide dismutase (1 U/mL), scavenged O_2^- produced by the HX/XO and PMS–NADH systems. By contrast MA at 1–100 μM, unlike allopurinol (10 μM), was unable to inhibit XO activity. MA at 1–100 μM did not modify resting tone or the contractile responses elicited by 1 μM phenylephrine or 1 μM phorbol 12-myristate 13-acetate in rat aorta. MA at 1–100 μM, like dexamethasone (100 μM), decreased iNOS mRNA levels in activated macrophages. At 100 μM, MA also reduced TNF-α mRNA levels, but increased TGF-β mRNA levels. These results thus indicate that MA is an O_2^- scavenger and that it inhibits expression of the iNOS and TNF-α genes, suggesting that it may be of potential value in the treatment of inflammatory and/or neurodegenerative disorders. In addition, the finding that MA enhances TGF-β gene expression suggests that this polyphenol might also be of value in the prevention of cancer, autoimmune disorders, atherosclerosis and coronary heart disease.

Keywords: Mangiferin; Superoxide anion; Nitric oxide; Cytokines; mRNA levels; Rat; Peritoneal macrophages

1. Introduction

At high concentrations all reactive species—including free radicals, radical-derived species and nonradical reactive species—are toxic for living organisms and damage all major cellular constituents [1]. On the contrary, at low concentrations, ROS, including nitric oxide (NO) and superoxide anion (O_2^-), play a crucial role as regulatory mediators in cellular signalling processes [2] and in many physiological functions such as regulation of vascular tone, monitoring of oxygen tension in the control of ventilation,

* Corresponding author. Tel.: +34-981-563100; fax: +34-981-593316.
E-mail address: mpleiro@usc.es (J.M. Leiro).

Abbreviations: DMEM, Dulbecco's Eagle medium; DTT, dithiothreitol; DX, dexamethasone; HX, hypoxanthine; IFN-γ, interferon-gamma; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MA, mangiferin; NBT, nitroblue tetrazolium; PE, L-phenylephrine hydrochloride; PMA, phorbol 12-myristate 13-acetate; PMS, phenazine methosulphate; ROS, reactive oxygen species; RT–PCR, retrotranscriptase–polymerase chain reaction; SOD, superoxide dismutase; TGF-β, tumour growth factor-beta; TNF-α, tumour necrosis factor-alpha; XO, xanthine oxidase.

0006-2952/03/$ – see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00041-8

signal transduction from membrane receptors, and erythropoietin production [3]. Another important physiological function of ROS is the phagocyte-dependent killing of pathogens during the respiratory burst, a process characterised by an increase in oxygen consumption, activation of a metabolic hexose monophosphate shunt, and the generation and release of chemically reactive oxygen metabolites [4]. Additionally, ROS generated either extracellularly or intracellularly through ligand–receptor interactions can function as signal transduction molecules to activate cytokine production and modulate genome expression by specific and precise mechanisms during cell activation [5]. An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischaemia/reperfusion injury, obstructive sleep apnea, and other diseases [6]. Currently, there is increasing interest in therapeutic use of antioxidants to prevent tissue damage induced by overproduction of ROS, by reducing free radical formation or by scavenging or promoting the breakdown of these species [1,7,8]. Experiments in different *in vitro* and *in vivo* systems have demonstrated the potent antioxidant action of plant polyphenols [9], and it has been suggested that they can prevent oxidative-stress-related diseases [10,11]. Recently, the polyphenol mangiferin (MA), a C-glucosylxanthone, specifically 1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside, has attracted considerable interest in view of its numerous pharmacological activities, including antitumour and antiviral [12–15], antidiabetic [16–18], anti-bone resorption [19] and antioxidant activity [20,21]. In previous studies, we have demonstrated that MA inhibit ROS production in rat peritoneal macrophages stimulated *in vitro* with LPS, IFN-γ or PMA [22]; however, little is known about the molecular bases of these inhibitory effects, or about the effects of MA on other important targets of oxidant stress, such as the vascular endothelium, whose dysfunction plays a critical role in the pathophysiology of several vascular diseases [23], including for example heart failure [24].

In the present study, we investigated the molecular bases of the effects of MA, firstly in *in vitro* studies to assess whether it scavenges O_2^- and/or modulates XO activity, and secondly in studies of effects on production of mRNAs of iNOS, TNF-α and TGF-β in activated rat peritoneal macrophages. Additionally, and in view of the antioxidant activity of MA, we also investigated its possible regulatory effect on vascular tone in rat aortic rings.

2. Materials and methods

2.1. Animals

Male Wistar rats (age: 8–10 weeks) (Iffa-Credo), purchased from Criffa (Barcelona, Spain), were used. They were housed (groups of five) in Makrolon cages (Panlab) on poplar shaving bedding (B&K Universal) in a standard bio-clean animal room, illuminated from 8:00 to 20:00 hr (12 hr light:12 hr dark cycle) and maintained at a temperature of 22–24°. The animals had free access to food pellets (B&K Universal), and to tap water, and were allowed to acclimatise for 1 week before the experiments.

2.2. Ethical approval

All experiments were carried out in accordance with European regulations on animal protection (Directive 86/609), the Declaration of Helsinki, and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela.

2.3. Stimuli, drugs and chemicals

Stock solution of LPS from *Escherichia coli* serotype 0111:B4 (Sigma Chemical Co.) was made up at 10 mg/mL in phenol-red-free DMEM (Sigma) and stored at −20° until use. Thioglycollate broth (Merck) was prepared to a concentration of 3% (30 mg/mL) in PBS, autoclaved at 121° for 10 min, and stored at room temperature until use. MA, DX, L-PE, PMA, xanthine, SOD (from bovine erythrocytes), XO (from buttermilk), HX, NBT, β-nicotinamide adenine dinucleotide (NADH), PMS, acetylcholine hydrochloride and allopurinol were likewise purchased from Sigma, and recombinant murine IFN-γ from Genzyme. The appropriate dilutions of the above drugs were prepared everyday immediately before use, in phosphate buffer (for the experiments involving the HX–XO system) or in deionised water (for other experiments), from the following concentrated stock solutions (100 mM unless otherwise specified) kept at −20°: MA and PMA in DMSO (Sigma); PE in deionised water (sodium bisulfite 40 mM was added to the PE stock solution to prevent oxidation); acetylcholine (to test the presence and integrity of the endothelium), allopurinol, and SOD (20 kU/mL) in deionised water; HX and xanthine (10 mM) in a 10 mM potassium hydroxide solution.

XO was dissolved daily before the experiments in a phosphate buffer. In all tests, deionised water and the appropriate dilutions of the different vehicles used had no significant pharmacological effects. All chemicals were of analytical grade.

2.4. Isolation and activation of rat peritoneal-exudate macrophages

For induction of inflammatory responses, rats were injected intraperitoneally with 1 mL of 3% thioglycollate broth, and peritoneal exudate was extracted 5 days later.

Rat resident and inflammatory peritoneal macrophages were obtained as previously described [25,26]. Briefly, the abdomen of the rat was soaked with 70% ethanol for disinfection, a midline incision was then made with scissors, and the abdominal skin retracted. Thirty milliliter of DMEM was then injected into the peritoneal cavity using a syringe with a 19-G needle. After gentle abdominal massage, about 30 mL of peritoneal fluid was extracted using the same syringe and transferred to 50-mL sterile polypropylene tubes on ice. A 20-μL aliquot was then extracted for cell counting in a hemocytometer and the cells were washed once by centrifugation at 400 *g* and resuspended to a concentration of 10^6 cells/mL. The number of viable cells were estimated by the trypan blue exclusion test. Aliquots of 100 μL of the cell suspension were added to the wells of 96-well microculture plates (Corning, USA) and left for 90 min in a humidified incubator (37°, 5% CO_2) to allow adhesion. Non-adherent cells were then removed by gently washing with DMEM. More than 97% of the adherent cells showed nonspecific esterase activity, indicating that they were macrophages [26].

2.5. Determination of XO activity by use of the xanthine–XO system

The potential effect of MA on XO activity was investigated by measuring uric acid formation, as previously described [27]. Briefly, 1-mL test solutions of phosphate buffer (50 mM KH_2PO_4–KOH, pH 7.4) containing MA at different concentrations (1, 10 or 100 μM), EDTA-Na_2 (1 mM), and 0.066 U of XO were incubated for 15 min at room temperature. The reaction was started by adding xanthine in phosphate buffer (final concentration: 100 μM), and the rate of uric acid production was estimated from the difference in absorbance at 295 nm (measured at room temperature for 10 min in a UV-Vis absorption spectrophotometer, Shimadzu UV-240) between the test solution and a blank solution in which XO was replaced by buffer solution. In some assays, the validity of the method was confirmed by assessing the influence of allopurinol (a well-known XO inhibitor) on uric acid formation.

2.6. Generation of O_2^- by use of the HX–XO system

The O_2^- was generated enzymatically in an HX–XO system and quantified by the spectrophotometric measurement of the product of the reduction of NBT, essentially following the procedure described previously [27,28]. The 250-μL test solutions contained the following, in phosphate buffer (50 mM KH_2PO_4–KOH, pH 7.4): 1 mM EDTA-Na_2, 100 μM NBT, and MA at various concentrations (1, 10 or 100 μM). Control experiments were carried out simultaneously using the same test solution but without MA. The reaction was started with the test solution already in a Cobas Fara 22-3123 AutoAnalyzer (Roche), by adding XO in phosphate buffer (final concentration: 0.066 U/mL) and continued at room temperature (22–24°) for 10 min. The rate of NBT reduction was estimated from the difference in absorbance at 560 nm between the test solution and a blank solution in which the XO was replaced by buffer. In some experiments, the sensitivity of the method was evaluated by assessing the influence of SOD (1 U/mL), a known scavenger of O_2^-.

2.7. Generation of O_2^- by use of the PMS–NADH system

O_2^- was also generated in a non-enzymatic PMS–NADH system and again quantified by the spectrophotometric measurement of the product of the reduction of NBT, essentially following the procedure described previously [28]. We used 300-μL test solutions made up in phosphate buffer (50 mM KH_2PO_4–KOH, pH 7.4) and containing 166 μM NADH, 43 μM NBT, 10 U/mL of SOD and MA at various concentrations (1, 10 or 100 μM). Control experiments were carried out simultaneously without MA. In addition, the possible capacity of MA to directly reduce NBT was determined by adding it to solutions containing only NBT in a phosphate buffer.

The reaction was started with test solutions already in a Cobas Fara 22-3123 (Roche) autoanalyser, by adding 2.7 mM PMS (freshly diluted in 100 mL of the above phosphate buffer) and continued at 25° for 10 min, a period over which absorbance increased linearly from the third minute. The rate of NBT reduction was calculated from the difference in absorbance at 560 nm with respect to a blank solution in which PMS was replaced by buffer solution, and was expressed as increment of absorbance per min.

2.8. Reverse transcription–polymerase chain reaction (RT–PCR)

One hundred microliter aliquots of 10^5 peritoneal inflammatory cells prestimulated *in vivo* 5 days previously with thioglycollate were incubated for 2 hr at 37° in DMEM containing 10 U/mL of IFN-γ, 100 ng/mL of LPS and MA at various concentrations (1, 10 and 100 μM) or without MA (controls); additionally, in some experiments for analysis of iNOS expression, the inhibitor of iNOS mRNA synthesis DX was added at 100 μM. Reverse transcription (RT) and polymerase chain reaction amplification (PCR) to detect RNA without any need for prior RNA extraction were performed as described by [29], with some modifications. Briefly, 10^5 peritoneal macrophages (obtained after prestimulation with thioglycollate as indicated above) were resuspended in 100 μL of freezing solution (0.15 M NaCl, 10 mM Tris, pH 8.0). Ten microliter of 2× RNase inhibitor (0.15 M NaCl, 10 mM Tris, pH 8.0, RNase inhibitor (100 U/μL), 5 mM DTT) was added to 10 μL of this cell suspension, and the resulting mixture was immediately frozen at −70°. The cells were subsequently defrosted rapidly in a room-temperature water bath, and total RNA was amplified by two-

step RT–PCR. cDNA synthesis was accomplished using 1.25 μM of random hexamer primers (Applied Biosystems), 250 μM of each deoxyribonucleotide triphosphate (dNTPs), 10 mM DTT, 10 U/20 μL of RNase inhibitor, 2.5 mM of $MgCl_2$ and 15 U of MultiScribe (murine leukaemia virus) reverse transcriptase (Applied Biosystems) in 30 mM Tris and 20 mM KCl, pH 8.3. The cycling parameters for the RT step are: hybridisation for 10 min at 25° and reverse transcription for 12 min at 42°. Twenty microliter of RT reaction mixture was amplified using a specific rat iNOS forward/reverse primer pair: 5′-TGGAAGCCGTAACAAAGGAAA-3′/5′-ACCACTCGTACTTGGGATGCT-3′ (selected from the complete sequence of Sprague–Dawley *Rattus norvegicus* iNOS mRNA, published in [30] and deposited in the GenBank of the National Center for Biotechnology Information (NCBI) accession number: U03699). Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the forward/reverse primer pair: 5′-ACCGCATCTTCTTGTGCAGT-3′/5′-GCCAAAGTTGTCATGGATGA-3′ [31] (NCBI, accession number: AF106860) was performed as a control of gene expression. These primers amplified a 563-bp (iNOS) or 544-bp (GAPDH) fragment. The 50-μL optimised reaction mixture contained reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 9.0), 0.2 mM of each dNTP, 0.4 μM of each primer and 1.5 U of rTaq DNA polymerase (Roche). Thermal cycling in an automatic thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer, Norwalk, USA) was as follows: initial denaturing at 94° for 5 min; then 35 cycles at 94° for 30 sec, 55° (iNOS) or 53° (GAPDH) for 45 sec, and 72° for 1 min; and finally a 7-min extension phase at 72°. In all experiments we performed RT–PCR controls without RNA or without reverse transcriptase; in no case were amplification products obtained. PCR products (20 μL aliquots) were separated on a 2% agarose gel in TBE buffer stained with 0.5 μg/mL of ethidium bromide, and photographed with a digital camera under a Spectroline 312 variable-intensity UV transilluminator (Spectroline) as previously described [32].

2.9. Multiplex RT–PCR (RT–MPCR)

RT–MPCR provides a very sensitive and accurate method for measuring expression levels of multiple genes [33]. cDNA was obtained by RT as described above, and cleared by phenol extraction and ethanol precipitation. MPCR was done using a CytoXpress™ kit (Rat Inflammatory Set 1, Biosource International) following the manufacturer's instructions. This kit is designed to direct the simultaneous amplification of specific ORF regions of the rat GAPDH, GM-CSF, TNF-α, IL-1β, IL-6 and TGF-β genes. A 50-μL MPCR mixture was made up with 10.5 μL of distilled water, 5.0 μL of 10× MPCR buffer, 5.0 μL 10× MPCR enhancer, 5.0 μL of 10× MPCR primers, 4.0 μL of dNTP (3.12 mM), 0.5 μL of rTaq DNA polymerase (5 U/μL) (Roche) and 20 μL of clean cDNA. Thermal cycling was as follows: initial denaturing at 96° for 1 min; 2 cycles with a denaturing step of 96° for 1 min and annealing step of 60° for 4 min; then 33 cycles at 94° for 1 min and 60° for 2.5 min; and finally a 10-min extension phase at 70°, followed by a soak at 25°. MPCR products (10-μL aliquots) were again analysed by 2% agarose gel electrophoresis, with ethidium bromide staining. The gel was photographed with a digital camera under a UV transilluminator, and the bands were quantified in TIF images using densitometry analysis software (ImageMaster Total Lab, version 2.00; Amersham-PharmaciaBiotech). The results were expressed as the ratio between the optical density (OD) of the band corresponding to the cytokine studied and the OD of the band corresponding to the control gene GAPDH.

2.10. DNA quantification

DNA concentration in PCR samples was assessed using the PicoGreen® dsDNA quantification assay (Molecular Probes), an ultrasensitive fluorescent nucleic acid stain for quantifying double-stranded DNA (dsDNA) in solution, following the instructions of the manufacturer. Calibration was against a standard curve of purified bacteriophage lambda DNA over the range 25 pg/mL–25 ng/mL. Fluorescence was measured with a microplate fluorescence reader (Bio-Tek Instruments) with excitation at 480 nm and emission at 520 nm.

2.11. ELISA for quantification of TNF-α

One hundred microliter aliquots of the macrophage suspension (10^6 peritoneal macrophages per mL, obtained after prestimulation with thioglycollate as indicated above) were incubated for 5 hr with LPS (100 ng/mL in DMEM) or LPS plus IFN-γ (10 U/mL in DMEM), and MA at various concentrations (1, 10 and 100 μM in DMEM) or without MA (controls) in 96-well microculture plates at 37° under 5% CO_2 in a humidified incubator. The plates were centrifuged at 400 *g* for 5 min and TNF-α was quantified in the supernatant using a sandwich ELISA developed by R&D Systems. PVC microtitre plates (Costar) were coated with 100 μL per well of capture antibody (goat anti-rat TNF-α) at 0.8 μg/mL in PBS by incubation overnight at room temperature. The plates were then washed five times with wash buffer (0.05% Tween 20 in PBS, pH 7.2–7.4) and blocked by adding 300 μL per well of block buffer (1% BSA, 5% sucrose in PBS with 0.05% NaN_3) and incubated for 2 hr at 37°. After a washing step (five times with wash buffer) and the addition of 100 μL of culture supernatant (see above), or standard solution (seven serial 2-fold dilutions of a 2000 pg/mL stock solution of recombinant rat TNF-α in reagent diluent, i.e. PBS containing 1% BSA, pH 7.2–7.4), the plates were then incubated for 2 hr with detection antibody (biotinylated goat anti-rat TNF-α) at 300 ng/mL in reagent diluent. Plates were again washed five times with wash buffer, adding 100 μL of a 1/200 working dilution of

streptavidin conjugated to horseradish peroxidase for 20 min at room temperature. After washing with wash buffer, peroxidase activity was detected by adding 100 μL of substrate solution (*o*-phenylenediamine (Sigma) at a concentration of 0.04% in phosphate–citrate buffer (pH 5.0) containing 0.001% H_2O_2). After 20 min incubation at room temperature, the reaction was stopped with 25 μL of 3 N H_2SO_4, and OD at 492 nm was measured with an ELISA reader (Titertek Multiscan, Flow Laboratories).

2.12. Functional (contraction/relaxation) studies in isolated rat aorta rings

Vascular rings were prepared from the aortas of male Wistar rats weighing 230–270 g, essentially as described elsewhere [34]. In addition, contraction studies were performed following the general procedure described in [35]. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (1 μM) to induce more than 50% relaxation of rings pre-contracted with PE (1 μM). Vessels were considered to be denuded of functional endothelium when there was no relaxation response to acetylcholine.

2.13. Activity in pre-contracted rat aortic rings and effects on resting tension

After an equilibration period of at least 1 hr, isometric contractions induced by PE (1 μM) and PMA (1 μM) were obtained. When the contraction of the tissue in response to the corresponding vasoconstrictor agent had stabilised (after approximately 20 min for PE or 60 min for PMA), increasing cumulative concentrations of MA (1–100 μM) were added to the bath at 15–20 min intervals. Control tissues were subjected to the same procedures simultaneously, but omitting the drug and adding the vehicle (i.e. an appropriate DMSO dilution).

In another series of experiments, different concentrations of MA (1–100 μM) were added to baths containing non-contracted rat aortic rings, in order to analyse the effects of this compound on resting vascular tone.

2.14. Statistics

Data are expressed as means ± SEM. Statistical significances ($P = 0.05$) were determined by one-way ANOVA followed by the Tukey–Kramer test for multiple comparisons.

3. Results

3.1. O_2^- scavenging activity of MA

To determine whether the inhibitory action of MA on ROS production in rat macrophages [22] is due to a ROS-scavenger effect, the ability of MA to scavenge O_2^- generated by an enzymatic (HX–XO) or non-enzymatic system (PMS–NADH) was investigated. MA at 1–100 μM was capable of removing O_2^- generated by the enzymatic system (Fig. 1A) with an IC_{50} value of 8.2 ± 0.72 μM (N = 5); whereas concentrations of 10–100 μM (IC_{50} of 31.8 ± 1.85 μM; N = 5) were necessary to effectively scavenge the O_2^- generated by the non-enzymatic system (Fig. 1B). The MA concentration required to scavenge O_2^- to the same extent as 1 U/mL SOD (a known O_2^- scavenger) was 100 μM.

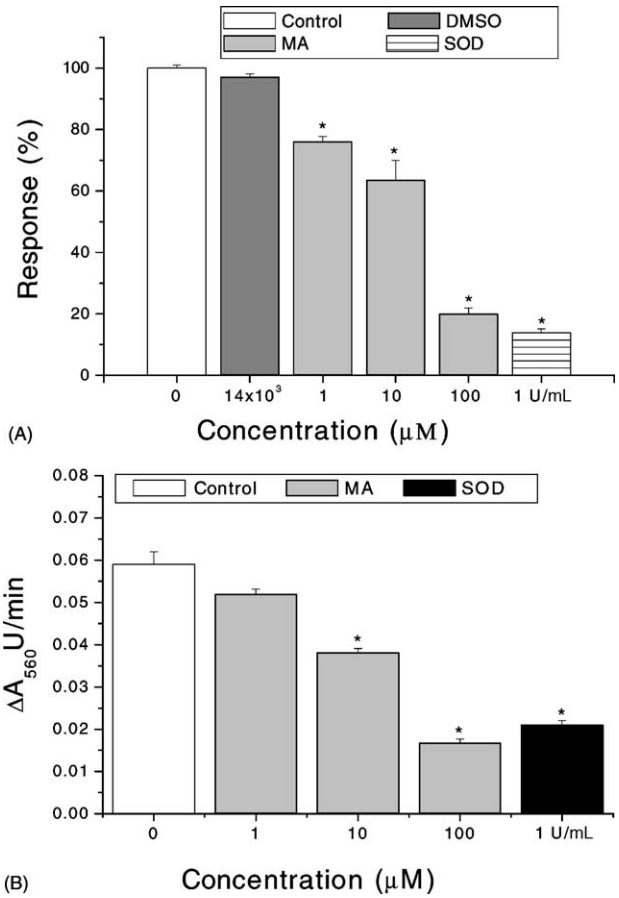


Fig. 1. Effects of MA (1–100 μM) and SOD (1 U/mL) on reduction of NBT by O_2^- generated (A) by XO in the presence of 100 μM HX and (B) in the PMS–NADH system. The O_2^- scavenger activity of MA and SOD in the enzymatic system HX–XO (A) is expressed as percentage absorbance at 560 nm with respect to the untreated control. Bars represent means ± SEM (N = 5). Asterisks (*) indicate statistical significance ($P < 0.01$) with respect to control (without treatment).

3.2. Effects of MA on XO activity

To rule out possible inhibitory effects of MA on XO activity, we monitored its effects on the XO-catalysed production of uric acid from xanthine. As shown in Fig. 2, MA at 1–100 μM did not significantly affect XO activity. In contrast, the inhibitory reference drug allopurinol at 10 μM inhibited uric acid generation, indicating that it reduced XO activity (Fig. 2).

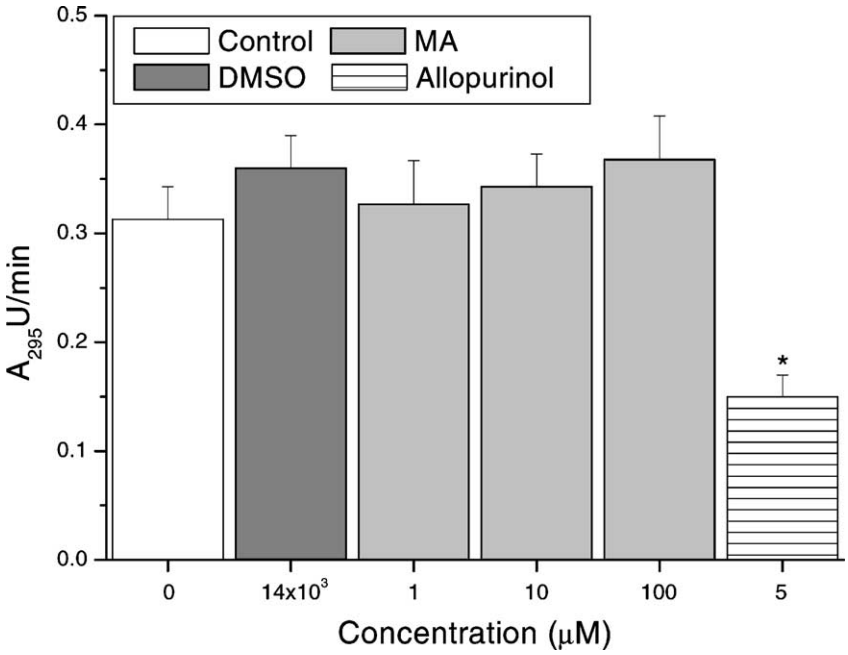
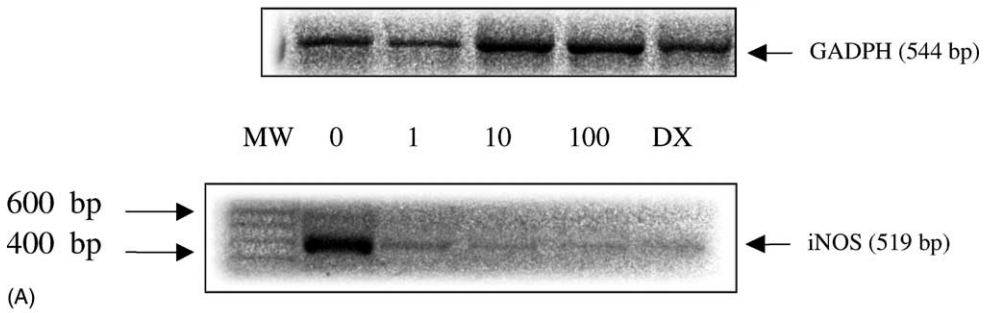


Fig. 2. Effects of MA (1–100 μM) and allopurinol (5 μM) on XO-catalysed production of uric acid in the presence of 100 μM of xanthine. Values shown are means ± SEM (N = 5). Asterisk (*) indicates statistical significance ($P < 0.01$) with respect to control.



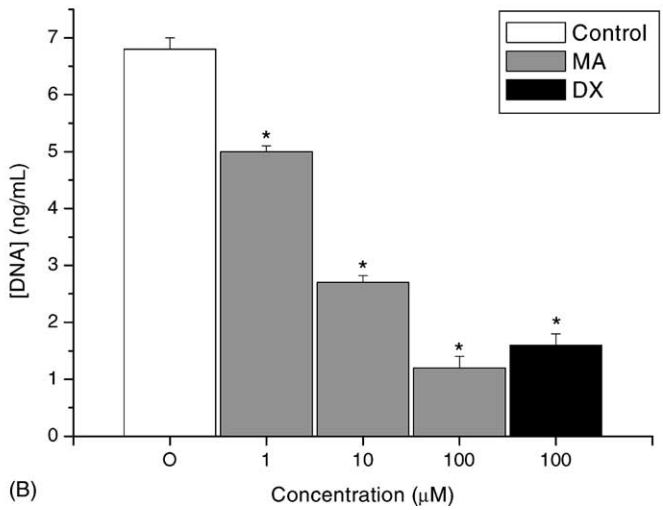


Fig. 3. *In vitro* effects of MA (1–100 μM) and DX (100 μM) on levels of iNOS mRNAs in rat peritoneal macrophages, as determined by semiquantitative RT–PCR. Macrophages were prestimulated *in vivo* with 3% thioglycollate broth i.p. and stimulated *in vitro* with LPS (100 ng/mL) and IFN-γ (10 U/mL), in the absence or presence of MA (1–100 μM) or DX (100 μM). (A) 2% agarose analysis of RT–PCR products using primers for iNOS (519 bp) or the control gene GADPH (544 bp) and (B) DNA quantification of RT–PCR products by the fluorescent nucleic acid stain PicoGreen® in ng/mL. Bars show means ± SEM (N = 3). Asterisks (*) indicate statistical significance ($P < 0.01$) with respect to control. MW: molecular weight markers (50-bp ladder).

3.3. Effects of MA on iNOS mRNA levels

In order to investigate whether the suppression of iNOS activity in rat macrophages by MA [22] is due to reduced iNOS mRNA synthesis, iNOS mRNA levels were determined by a semiquantitative RT–PCR in total RNA samples extracted from thioglycollate-prestimulated rat peritoneal macrophages. The amplification of cDNA with primers specific for rat iNOS and GAPDH (as constitutive gene) is shown in the Fig. 3. The results indicate that significantly lower levels of iNOS mRNA are present in rat peritoneal macrophages incubated *in vitro* with LPS and IFN-γ in the presence of MA than in the absence of MA (Fig. 3). MA and the glucocorticoid DX at a concentration of 100 μM show similar inhibitory effects on iNOS mRNA levels (Fig. 3).

3.4. Effects of MA on inflammatory cytokine mRNA levels

To assess the effect of MA on the synthesis of mRNAs of cytokines involved in inflammation, a semiquantitative RT–MPCR was used. Macrophages prestimulated *in vivo* with the inflammatory agent sodium thioglycollate and then incubated *in vitro* for 2 hr with LPS and IFN-γ show high levels of mRNAs of the cytokines TGF-β and TNF-α (Fig. 4). MA dose-dependently increased TGF-β mRNA levels and inhibited TNF-α mRNA levels, but did not affect mRNA levels of GAPDH, used as gene expression control (Fig. 4).

3.5. Effects of MA on in vitro TNF-α production

To confirm that the apparent inhibitory effect of MA on TNF-α mRNA production leads to reduced secretion of

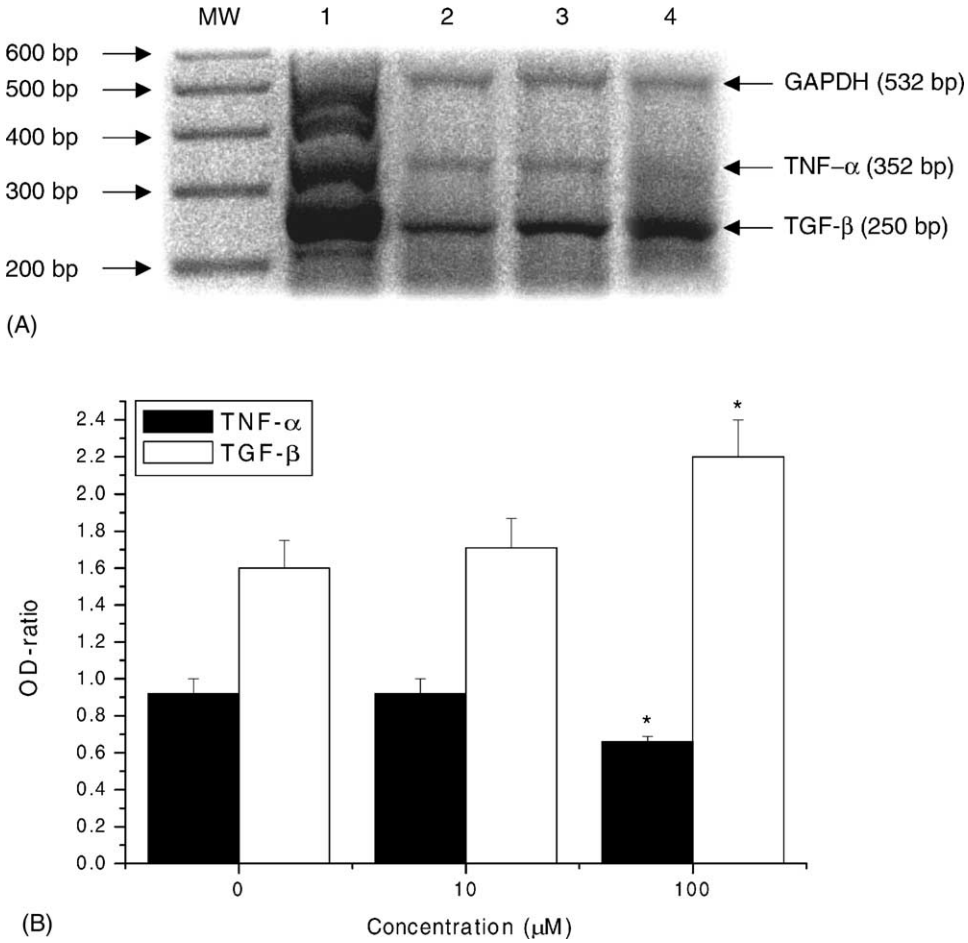


Fig. 4. Effects of MA (10–100 μM) on mRNA levels of cytokines TNF-α and TGF-β in rat peritoneal macrophages as determined by semi-quantitative multiplex RT–PCR (RT–MPCR). Macrophages were prestimulated *in vivo* with 3% thioglycollate broth (i.p.) and stimulated *in vitro* with LPS (100 ng/mL) and IFN-γ (10 U/mL), in the absence or presence of MA (10–100 μM). (A) Samples were run on a 2% agarose gel and (B) mRNA l evels were quantified by densitometric analysis using image analysis software. (A) MW: molecular weight markers (100 bp ladder). *Lane 1*: Results for reaction mixture containing specimen cDNA control, supplied with the kit, and the primers used (Biosource CytoXpress Rat Inflammatory Cytokines Set 1, comprising primers for GM-CSF (210 bp), TGF-β (250 bp), IL-1β (295 bp), TNF-α (352 bp), IL-6 (453 bp) and GAPDH (532 bp). *Lane 2*: Results for reaction mixture containing cDNA from rat macrophages incubated without MA (control). *Lanes 3 and 4*: Results for reaction mixtures containing cDNA from rat macrophages incubated with 10 or 100 μM respectively. (B) Bars are means ± SEM (N = 3) of [OD of cytokine band]/[OD of corresponding band in analysis using primer for GAPDH], where OD = optical density. (*) Significantly different from control, $P < 0.01$.

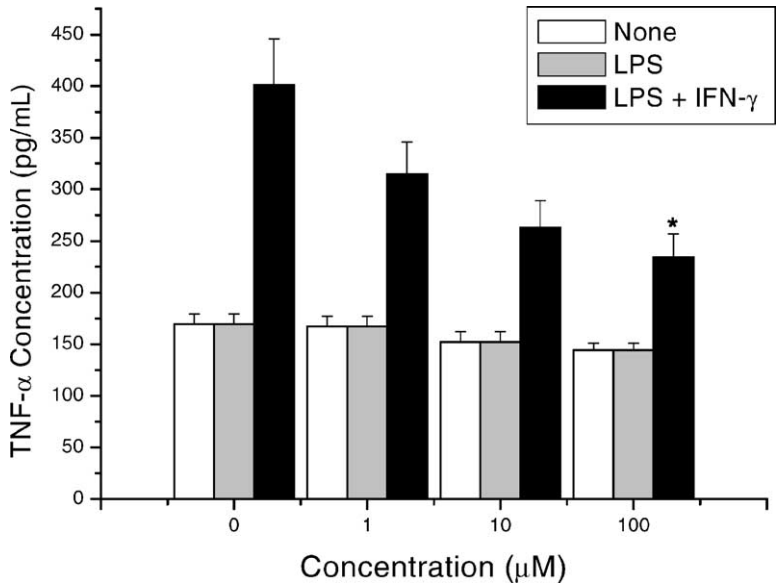


Fig. 5. Effects of MA (1–100 μM) on TNF-α levels (as determined by sandwich ELISA) in culture supernatant of peritoneal macrophages prestimulated *in vivo* with 3% thioglycollate broth (i.p.) and stimulated *in vitro* for 5 hr with LPS (100 ng/mL) or LPS (100 ng/mL) and IFN-γ (10 U/mL). Bars represent means ± SEM (N = 3). Asterisk (*) indicates statistical significance ($P < 0.05$) with respect to control (i.e. the corresponding no-MA treatment).

TNF-α by macrophages, we used a sandwich ELISA to measure TNF-α levels in culture supernatants of macrophages prestimulated *in vivo* with thioglycollate and then incubated *in vitro* for 5 hr with LPS or LPS plus IFN-α. Macrophages incubated with LPS plus IFN-α (unlike macrophages incubated with LPS alone) showed significantly higher TNF-α production than control macrophages, and MA dose-dependently inhibited TNF-α production by LPS/IFN-α-treated macrophages, the inhibition reaching statistical significance at 100 μM MA (Fig. 5).

3.6. Effects of MA on resting tension in rat aorta and on pre-contracted rat aortic rings

In our experiments the rat aortic rings lacked spontaneous activity, as we have previously reported [34]. Resting tone was unaffected by DMSO (14 mM–14 μM) and MA (1–100 μM) in intact rat aortic rings (N = 5, $P > 0.05$, data not shown).

PE (1 μM) and PMA (1 μM) induced a slow and sustained contraction in isolated aortic rings with endothelium. The maximal tensions reached were 1957 ± 146 and 1826 ± 138 mg, respectively (N = 5). These contractile effects were maintained without significant tension changes for at least 90 min. Neither MA (1–100 μM) nor DMSO (14 mM–14 μM) had significant effects on either the PE- or the PMA-induced contractions (N = 5, $P > 0.05$ in all cases) (data not shown).

4. Discussion

ROS and reactive nitrogen species (RNS) play a central role in many cell processes, and the balance between NO and O_2^- is a critical determinant in the aetiology of many human diseases including atherosclerosis, neurodegenerative disease, ischaemia-reperfusion and cancer [36]. Leucocytes, including macrophages and eosinophils, participate in the innate immune response by phagocytising foreign particles and producing cytokines, ROS and RNS involved in the destruction of pathogens [21]. The overproduction of ROS and RNS by activated phagocytic cells has been implicated in many inflammatory disorders associated with various pathologies [37]. Previously, we have demonstrated that the polyphenol MA is capable of modulating the activation and functionality of rat macrophages, through partial inhibition of chemotactic migration, phagocytic activity, and ROS and RNS production [22], but until now little was known about the molecular processes involved in its antioxidant activity. In this study we demonstrate that MA possesses potent O_2^--scavenger activity, similar to that of other natural polyphenols [28,38]. The major sources of O_2^- *in vivo* include mitochondrial respiration, membrane-associated NADH–NADPH oxidases, and the enzyme XO which catalyses the formation of O_2^- and H_2O_2 from oxygen and xanthine or HX [36,39,40]. Our results demonstrate that MA does not affect XO activity, which confirms that its inhibitory effect on ROS production is fundamentally due to O_2^- scavenging.

NO is a short-lived free radical gas that has a variety of functions including vasodilation, neurotransmission, and tumouricidal and microbicidal activities [41]. NO was first identified as the endothelial-derived relaxing factor (EDRF), and its role in the signalling pathway leading to this physiological effect was rapidly established [40]. NO is produced in large amounts by macrophages, and we have previously demonstrated that MA inhibits NO production

by thioglycollate-elicited rat macrophages [22]. In this study we investigated whether this inhibition is related to modulation of the level of expression of the gene for NO synthase (isozyme NOS-II, NOS2 or iNOS), which in mammals can be induced in macrophages by cytokines, LPS and a variety of other agents (including ROS) [42]. Our results indicate that MA dose-dependently inhibits iNOS mRNA production in peritoneal exudate macrophages stimulated *in vivo* with thioglycollate and then *in vitro* with LPS and IFN-γ, and that the inhibition is similar to that caused by DX, a known suppressor of iNOS mRNA production [43]. A number of polyphenolic phytochemicals that also shown potent anti-oxidant activity can inhibit iNOS gene expression and NO generation in several types of cell, including macrophages [44–49]. The major agents inducing expression of iNOS in macrophages and other cells are LPS and cytokines, such as IL-1, IFN-γ and TNF-α [50]. In the present study MA dose-dependently inhibited the production of TNF-α by thioglycollate-elicited rat peritoneal macrophages stimulated with LPS and IFN-γ. Other polyphenols, such as green tea polyphenols, dose-dependently inhibit TNF-α gene expression in BALB/3T3 cells treated with okadaic acid [51,52]. Several cytokines including TGF-β are known to prevent the induction. The three isoforms of TGF-β are inhibitors of iNOS in mouse macrophages and renal epithelial cells, and in rat vascular smooth-muscle cells [53–58]. In the present study we demonstrated that MA increases TGF-β mRNA levels in peritoneal macrophages. TGF-β is known to be a powerful immunomodulatory agent, and has been shown to play a role in ablating the respiratory burst of activated macrophages [59] and inhibiting their ability to produce NO [60]. The interaction between iNOS and TGF-β may be a central homeostatic mechanism in mammalian physiology, with implications for a variety of human diseases associated with prolonged production of large amounts of NO [57]. Recent studies have implicated the TGF-β response system as a novel tumour-suppressor pathway in epithelial tissues [61], and as a suppressor of atherogenesis in blood vessels [62]. Alterations in the gene expression of TGF-β, particularly the TGF-β1 isoform, have been implicated in many disease processes: the total absence of TGF-β1 leads to an autoimmune-like syndrome [63], while decreased TGF-β1 levels have been linked to coronary artery disease [64].

An important physiological function of NO and O_2^- is the regulation of vascular tone [3]. Increasing evidence suggests that oxidant stress is involved in modulation of many endothelium functions and states, including vasomotor tone; the application of specific therapies to prevent ROS production may therefore help to amend endothelial dysfunctions in cardiovascular diseases [65,66]. In particular, and in view of their antioxidant properties, the polyphenols have been postulated as potentially protective against cardiovascular disease [27,67]. In the present study, the polyphenol MA did not modify resting tone or the contractile responses elicited by PE or PMA in rat aorta. In the aorta, a tenuous balance exists between the steady-state levels of NO and O_2^- [68], and these molecules have opposing effects on vascular tone and chemically react with each other in a way that negates their individual effects and leads to the production of potentially toxic substances, such as peroxynitrite ($ONOO^-$) [69]. The therapeutic aim is to increase NO (i.e. vasodilator) bioavailability, either by increasing endothelial NO production or by reducing endothelial O_2^- (i.e. vasoconstrictor) production [70]. As is demonstrated in the present study, MA is unable to modulate NO/O_2^- balance in vascular endothelium, and thus does not modulate endothelium-dependent vascular relaxation.

In conclusion, MA is a potent antioxidant molecule that acts primarily as an O_2^- scavenger without affecting the activity of the O_2^--generating enzyme XO. MA also modulates the gene expression of iNOS and of cytokines that regulate macrophage activity and participate directly in the regulation of NO production, namely TNF-α and TGF-β. These results suggest that MA might be of value in the treatment of immunopathological disorders characterised by overproduction of NO/O_2^-, such as inflammatory diseases, atherosclerosis or septic shock. In view of its antioxidant effects, MA may also be useful in the prevention of carcinogenesis, while its stimulatory effect on TGF-β, a cytokine inhibitor of angiogenesis [71], might be used to block tumour growth or protect against autoimmune diseases.

Acknowledgments

This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (CICYT) Spain, and the Consellería de Educación e Ordenación Universitaria, Xunta de Galicia, Spain.

References

[1] Young IS, Woodside JV. Antioxidants in health and disease. J Clin Pathol 2001;54:176–86.
[2] Remacle J, Raes M, Toussaint O, Renard P, Rao G. Low levels of reactive oxygen species as modulators of cell function. Mutat Res 1995;316:103–22.
[3] Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47–95.
[4] Ramarao N, Gray-Owen SD, Meyer TF. *Helicobacter pylori* induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity. Mol Microbiol 2000;38:103–13.
[5] Roebuck KA. Oxidant stress regulation of IL-8 and ICAM-1 gene expression: differential activation and binding of the transcription factors AP-1 and NF-kappaB [Review]. Int J Mol Med 1999;4:223–30.
[6] Toyokuni S. Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 1999;49:91–102.
[7] Collins AR. Oxidative DNA damage, antioxidants, and cancer. Bioessays 1999;21:238–46.
[8] Cuzzocrea S, Riley OP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev 2001;53:135–59.

[9] Damianaki A, Bakogeorgou E, Kampa M, Notas G, Hatzoglou A, Panagiotou S, Gemetzi C, Kouroumalis E, Martin PM, Castanas E. Potent inhibitory action of red wine polyphenols on human breast cancer cells. J Cell Biochem 2000;78:429–41.
[10] Aucamp J, Gaspar A, Hara Y, Apostolides Z. Inhibition of xanthine oxidase by catechins from tea (*Camellia sinensis*). Anticancer Res 1997;17:4381–5.
[11] Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. Tannins and human health: a review. Crit Rev Food Sci Nutr 1998;38:421–64.
[12] Zheng MS, Lu ZY. Antiviral effect of mangiferin and isomangiferin on herpes simplex virus. Chin Med J 1990;103:160–5.
[13] Guha S, Ghosal S, Chattopadhyay U. Antitumor, immunomodulatory and anti-HIV effect of mangiferin, a naturally occurring glucosylxanthone. Chemotherapy 1996;42:443–51.
[14] Yoosook C, Bunyapraphatsara N, Boonyakiat Y, Kantasuk C. Anti-*Herpes simplex* virus activities of crude water extracts of Thai medicinal plants. Phytomedicine 2000;6:411–9.
[15] Yoshimi N, Matsunaga K, Katayama M, Yamada Y, Kuno T, Qiao Z, Hara A, Yamahara J, Mori H. The inhibitory effects of mangiferin, a naturally occurring glucosylxanthone, in bowel carcinogenesis of male F344 rats. Cancer Lett 2001;163:163–70.
[16] Ichiki H, Miura T, Kubo M, Ishihara E, Komatsu Y, Tanigawa K, Okada M. New antidiabetic compounds, mangiferin and its glucoside. Biol Pharm Bull 1998;21:1389–90.
[17] Miura T, Ichiki H, Iwamoto N, Kato M, Kubo M, Sasaki H, Okada M, Ishida T, Seino Y, Tanigawa K. Antidiabetic activity of the rhizoma of *Anemarrhena asphodeloides* and active components, mangiferin and its glucoside. Biol Pharm Bull 2001;24:1009–11.
[18] Miura T, Ichiki H, Hashimoto I, Iwamoto N, Kato M, Kubo M, Ishihara E, Komatsu Y, Okada M, Ishida T, Tanigawa K. Antidiabetic activity of a xanthone compound, mangiferin. Phytomedicine 2001;8:85–7.
[19] Li H, Miyahara T, Tezuka Y, Namba T, Nemoto N, Tonami S, Seto H, Tada T, Kadota S. The effect of Kampo formulae on bone resorption *in vitro* and *in vivo*. I. Active constituents of Tsu-kan-gan. Biol Pharm Bull 1998;21:1322–6.
[20] Sánchez GM, Re L, Giuliani A, Núñez-Selles AJ, Davison GP, León-Fernández OS. Protective effects of *Mangifera indica* L. extract, mangiferin and selected antioxidants against TPA-induced biomolecules oxidation and peritoneal macrophage activation in mice. Pharmacol Res 2000;42:565–73.
[21] Moreira RR, Carlos IZ, Vilega W. Release of intermediate reactive hydrogen peroxide by macrophage cells activated by natural products. Biol Pharm Bull 2001;24:201–4.
[22] García D, Delgado R, Ubeira FM, Leiro J. Modulation of rat macrophage function by the *Mangifera indica* L. extracts Vimang and mangiferin. Int Immunopharmacol 2002;2:797–806.
[23] Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. Am J Physiol Cell Physiol 2001;280:C719–41.
[24] López-Farre A, Casado S. Heart failure, redox alterations, and endothelial dysfunction. Hypertension 2001;38:1400–5.
[25] Leiro J, Iglesias R, Paramá A, Sanmartín ML, Ubeira FM. Respiratory burst response of rat macrophages to microsporidian spores. Exp Parasitol 2001;98:1–9.
[26] Leiro J, Álvarez E, García D, Orallo F. Resveratrol modulates rat macrophage functions. Int Immunopharmacol 2002;2:767–74.
[27] Orallo F, Álvarez E, Camiña M, Leiro J, Gómez E, Fernández P. The possible implication of *trans*-resveratrol in the cardioprotective effects of long-term moderate wine consumption. Mol Pharmacol 2002;61:294–302.
[28] Álvarez E, Leiro J, Orallo F. Effect of (−)-epigallocatechin-3-gallate on respiratory burst of rat macrophages. Int Immunopharmacol 2002;2:849–55.
[29] Klebe RJ, Grant GM, Grant AM, García MA, Giambernardi TA. RT–PCR without RNA isolation. In: Siebert PD, Larrick JW, editors. Gene cloning and analysis by RT–PCR. Palo Alto, CA: BioTecniques Books; 1998. p. 57–68.
[30] Galea E, Reis DJ, Feinstein DL. Cloning and expression of inducible nitric oxide synthase from rat astrocytes. J Neurosci Res 1994;37:406–14.
[31] Zheng J, Ramirez VD. Isolation of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA isoform from rat brain by a rapid PCR-based cloning method and its expression by RT–PCR. National Center for Biotechnology Information (NCBI), accession number AF106860; 2000
[32] Leiro J, Siso MIG, Paramá A, Ubeira FM, Sanmartín ML. RFLP analysis of PCR-amplified small subunit ribosomal DNA of three fish microsporidian species. Parasitology 2000;120:113–9.
[33] Dostal DE, Kempinski AM, Motel TJ, Baker KM. Absolute quantification of messenger RNA using multiplex RT–PCR. In: Siebert PD, Larrick JW, editors. Gene cloning and analysis by RT–PCR. Natick, MA: BioTechniques Books; 1998, p. 71–90.
[34] Orallo F. Study of the *in vivo* and *in vitro* cardiovascular effects of a hydralazine-like vasodilator agent (HPS-10) in normotensive rats. Br J Pharmacol 1997;121:1627–36.
[35] Holland HL, Gu JX, Orallo F, Camiña M, Fabeiro P. Enantioselective synthesis and pharmacological evaluation of a new type of verapamil analog with hypotensive and calcium antagonist activities. Pharmaceut Res 1999;16:281–7.
[36] Darley-Usmar V, Wiseman H, Halliwell B. Nitric oxide and oxygen radicals: a question of balance. FEBS Lett 1995;369:131–5.
[37] Bubarkt V, Kolb H. Nitric oxide in the immunopathogenesis of type 1 diabetes. In: Mayer B, editor. Handbook of experimental pharmacology, Vol. 143. Nitric oxide. Berlin: Springer; 2000. p. 525–44.
[38] Kaul A, Khanduja KL. Plant polyphenols inhibit benzoyl peroxide-induced superoxide anion radical production and diacylglyceride formation in murine peritoneal macrophages. Nutr Cancer Int J 1999;35:207–11.
[39] Forman HJ, Torres M. Signaling by the respiratory burst in macrophages. IUBMB Life 2001a;51:365–71.
[40] Forman HJ, Torres M. Redox signaling in macrophages. Mol Aspects Med 2001;22:189–216.
[41] Nathan C, Xie QW. Nitric oxide synthase: roles, tolls and control. Cell 1994;78:915–8.
[42] Mendes AF, Carvalho AP, Caramona MM, Lopes MC. Diphenyleneiodonium inhibits NF-kappaB activation and iNOS expression induced by IL-1beta: involvement of reactive oxygen species. Mediators Inflamm 2001;10:209–15.
[43] Kleinert H, Euchenhofer C, Ihrig-Biedert I, Forstermann U. In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NF-kappaB. J Biol Chem 1996;271:6039–44.
[44] Lin YL, Lin JK. (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. Mol Pharmacol 1997;52:465–72.
[45] Virgili F, Kobuchi H, Packer L. Procyanidins extracted from *Pinus maritima* (Pycnogenol): scavengers of free radical species and modulators of nitrogen monoxide metabolism in activated murine RAW264.7 macrophages. Free Radic Biol Med 1998;24:1120–9.
[46] Xiong O, Tezuka Y, Kaneko T, Li H, Tran LQ, Hase K, Namba T, Kadota S. Inhibition of nitric oxide by phenylethanoids in activated macrophages. Eur J Pharmacol 2000;14:137–44.
[47] Chen Y, Yang L TJ. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-kappaB activation. Biochem Pharmacol 2000;59:1445–57.
[48] Luceri C, Caderni G, Sanna A, Dolara P. Red wine and black tea polyphenols modulate the expression of cycloxygenase-2, inducible nitric oxide synthase and glutathione-related enzymes in azoxymethane-induced f344 rat colon tumors. J Nutr 2002;132:1376–9.
[49] Tsai S-H, Lin-Shiau S-Y, Lin J-K. Suppression of nitric oxide synthase and the down-regulation of the activation of NFκB in macrophages by resveratrol. Br J Pharmacol 1999;126:673–80.

[50] Förstermann U. Regulation of nitric oxide synthase expression and activity. In: Mayer B, editor. Handbook of experimental pharmacology, Vol. 143. Nitric oxide. Berlin: Springer; 2000. p. 71–91.
[51] Suganuma M, Sueoka E, Sueoka N, Okabe S, Fujiki H. Mechanisms of cancer prevention by tea polyphenols based on inhibition of TNF-alpha expression. Biofactors 2000;13:67–72.
[52] Yang F, Oz HS, Barve S, de Villiers WJ, McClain CJ, Varilek GW. The green tea polyphenol (−)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6. Mol Pharmacol 2001;60:528–33.
[53] Ding A, Nathan CF, Graycar J, Derynck R, Stuehr DJ, Srimal S. Macrophage deactivating factor and transforming growth factors β1, β2 and β3 inhibit catalytic activity. Neuropharmacology 1990;33: 1245–51.
[54] Förstermann U, Schmidt HH, Kohlhaas KL, Murad F. Induced RAW 264.7 macrophages express soluble and particulate nitric oxide synthase: inhibition by transforming growth factor-beta. Eur J Pharmacol 1992;225:161–5.
[55] Schini VB, Durante W, Elizondo E, Scott BT, Junquero DC, Schafer AI, Vanhoutte PM. The induction of nitric oxide synthase activity is inhibited by TGF-beta1, PDGFAB and PDGFBB in vascular smooth muscle cells. Eur J Pharmacol 1992;216:379–83.
[56] Perrella MA, Yoshizumi M, Fen Z, Tsai J-C, Hsieh C-M, Kourembanas S, Lee M-E. Transforming growth factor-β1, but not dexamethasone, down-regulates nitric oxide synthase mRNA after its induction by interleukin-1β in rat smooth muscle cells. J Biol Chem 1994;269:14595–600.
[57] Vodovotz Y. Control of nitric oxide production by transforming growth factor-beta1: mechanistic insights and potential relevance to human disease. Nitric Oxide 1997;1:3–17.
[58] Miyajima A, Chen J, Kirman I, Poppas DP, Darracott-Vaughan EJR, Felsen D. Interaction of nitric oxide and transforming growth factor-beta1 induced by angiotensin II and mechanical stretch in rat renal tubular epithelial cells. J Urol 2000;164:1729–34.
[59] Tsunawaki S, Sporn M, Ding A, Nathan C. Deactivation of macrophages by transforming growth factor-β. Nature 1988;334: 260–2.
[60] Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan CF. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. J Exp Med 1993;178:605–13.
[61] Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. Cytokine Growth Factor Rev 1996;7:93–102.
[62] McCaffrey TA, Du B, Consigli S, Szabo P, Bray PJ, Hartner L, Weksler BB, Sanborn TA, Bergman G, Bush Jr HL. Genomic instability in the type II TGF-beta1 receptor gene in atherosclerotic and restenotic vascular cells. J Clin Invest 1997;100:2182–8.
[63] Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci USA 1993;90:770–4.
[64] Batuman O, Go D, Clark LT, Smith EL, Clements P, Feit A, Lederer D. Relationship between cytokine levels and coronary artery disease in women. Heart Dis 2001;3:80–4.
[65] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840–4.
[66] Carr A, Frei B. The role of natural antioxidants in preserving the biological activity of endothelium-derived nitric oxide. Free Radic Biol Med 2000;28:1806–14.
[67] Depeint F, Gee JM, Williamson G, Johnson IT. Evidence for consistent patterns between flavonoid structures and cellular activities. Proc Nutr Soc 2002;61:97–103.
[68] Somers MJ, Harrison DG. Reactive oxygen species and the control of vasomotor tone. Curr Hypertens Rep 1999;1:102–8.
[69] Munzel T, Hink U, Heitzer T, Meinertz T. Role for NADPH/NADH oxidase in the modulation of vascular tone. Ann NY Acad Sci 1999; 874:386–400.
[70] Britten MB, Zeiher AM, Schachinger V. Clinical importance of coronary endothelial vasodilator dysfunction and therapeutic options. J Int Med 1999;245:315–27.
[71] Mandriota SJ, Menoud P-A, Peppers MS. Transforming growth factor β1 down-regulates vascular endothelial growth factor receptor *2/flk-1* expression in vascular endothelial cells. J Biol Chem 1996;271: 11500–5.